Deubiquitinating Proteases in *Plasmodium falciparum*

A Senior Honors Thesis

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By

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ABSTRACT

Plasmodium falciparum is the blood-borne parasite responsible for the deadliest form of human malaria, a disease that kills up to two million people each year (1). Lack of effective vaccines and continued emergence of resistant strains demands the development of novel therapeutics for this continued global health problem (2, 3). In an effort to discover new drugs, we are investigating parasite expressed proteases of the ubiquitin-proteasome system as potential targets of therapeutic intervention. The ubiquitin-proteasome system regulates protein levels in all eukaryotic cells by covalent modification of protein substrates by ubiquitin and their subsequent direction to the proteasome for degradation (6). DUBs are proteases responsible for hydrolyzing the protein-ubiquitin bond; thus participating in multiple ubiquitin-related processes such as ubiquitin recycling and ubiquitin processing. Due to the increasing promise of proteases as drug targets for a variety of diseases and the importance of the ubiquitin-proteasome pathway in eukaryotes, we are interested in P. falciparum deubiquitinating proteases (PfDUBs) as putative drug targets (9).

Seventeen DUBs have recently been identified in the *P. falciparum* genome (11). Using BLASTP homology searching, we have determined that the DUBs USP14, UCH-L5, and POH1 appear to be conserved in *P. falciparum*. These three DUBs are known to associate with the proteasome in mammalian cells and we hypothesize that in *P. falciparum*, they serve a role in ubiquitin recycling through the removal of ubiquitin from protein substrates prior to entering the proteasome for degradation (21, 22, 24). Since the presence of monomeric ubiquitin is necessary for the initial conjugation of ubiquitin to

substrates, disruption of ubiquitin recycling may deplete the necessary pools of monomeric ubiquitin.

To validate the essentiality of these three PfDUBs, we have used molecular genetic techniques to insert onto the end of their coding regions a sequence encoding a gene that allows us to attenuate the expression of the resulting fusion protein. Regardless of protein attenuation, we show that parasite death occurs in minimal media as a result of the C-terminal modification of either PfUSP14 or PfPOH1. Contrary to our model, attenuation of these PfDUBs did not appreciably deplete monomeric ubiquitin; however the parasites do appear to show a possible stress-related response. In addition to our essentiality studies, we have biochemically characterized PfUCH-L5 by tagging it with a hemagglutinin (HA) epitope tag. We have immunoprecipitated PfUCH-L5-HA, assayed for deubiquitinating activity, and assessed its biochemical properties.

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To my parents, Edwin and Celedonia Mapa

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CHAPTER 1

INTRODUCTION

Plasmodium falciparum is the eukaryotic pathogen responsible for the deadliest form of human malaria, a devastating disease that continues to be a major global health concern in the 21st century. One-two million people die from malaria each year, making it the fifth leading cause of death from an infectious disease worldwide, and the World Health Organization (WHO) estimated up to 311 million cases in 2008 alone. About half of the world's population lives in areas at risk (Fig. 1.1) (1).

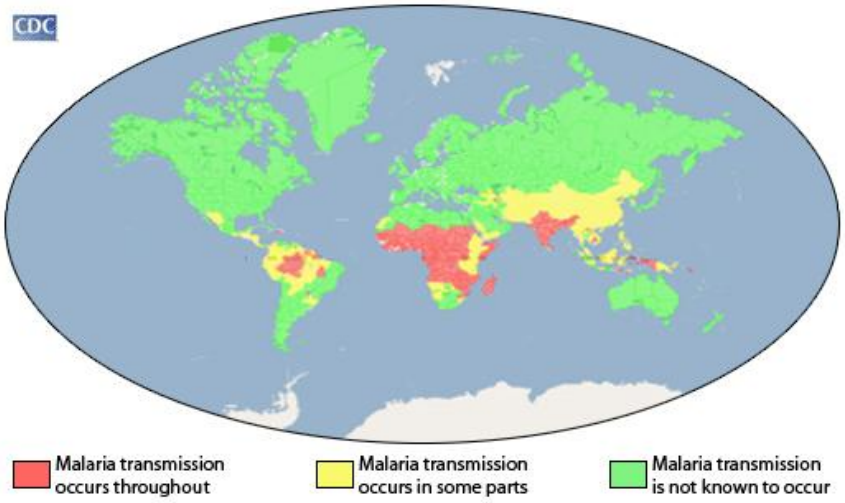

Figure 1.1 - Areas of the World Affected by Malaria (1**).**

P. falciparum is a parasitic protozoan transmitted between female *Anopheles* mosquitoes and humans. The parasite has a complex cycle in which meiosis occurs in the mosquito, with haploid forms in the humans hosts (Fig. 1.2). Briefly, during a blood meal, sporozoites from a malaria-infected mosquito migrate to and infect liver hepatocytes. The parasite replicates within the hepatocyte, eventually releasing hundreds of daughter merozoites into the circulatory system, which invade and infect erythrocytes, beginning what is termed the erythrocytic cycle. This portion of the life-cycle is

responsible for disease pathology through the continuous cycle of invasion and destruction of erythrocytes approximately every 48 hours. Up to 32 merozoites can emerge from a single infected erythrocyte. Concurrently, some of these erythrocyte stage parasites differentiate into sexual gametocytes which the mosquito takes up during a blood meal, thus transmitting the parasite back to the mosquito.

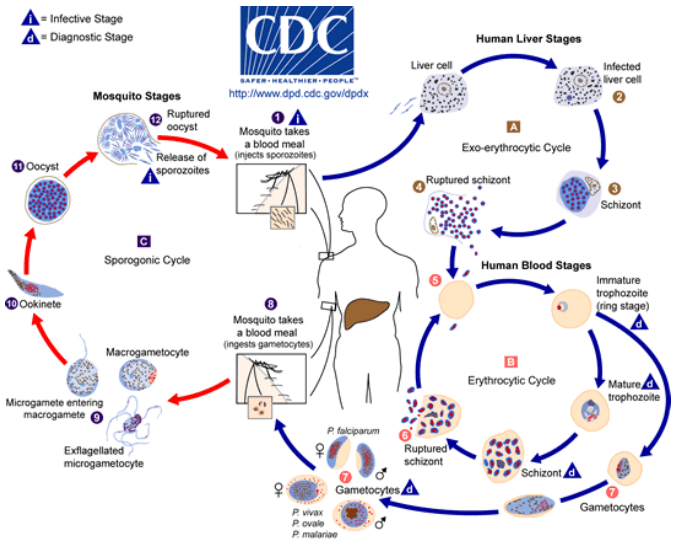

Figure 1.2 – Life cycle of *Plasmodium falciparum* (1). The sporogonic cycle consists of stages in the mosquito. The exo-erythrocytic cycle consists of the liver stages; the initial site of infection. The erythrocytic cycle is responsible for the disease causing stages of *P. falciparum*. Sexual gametocytes can differentiate from the erythrocytic stages and be transmitted back to mosquitoes in the sporogonic cycle.

Symptoms of the disease are similar to influenza and include fevers, chills, headaches, and body aches. A hallmark identifier is the recurring fever every 48 hours due to the synchronous rupture of the parasites during the erythrocytic cycle resulting in a massive cytokine response. Severe cases of malaria cause patients to suffer from anemia from erythrocyte hemolysis, blood coagulation, and cerebral-malaria, the most common cause of death (1).

Despite the availability of therapeutics, malaria continues to be a modern global health problem due to a wide range of factors. To date, no effective vaccine has been developed for malaria. Without even a promising vaccine in the pipeline, the

development of an effective vaccine is likely many years from fruition (2). A more pressing obstacle to the malaria problem is drug resistance. As of 2011, every known antimalarial has encountered some form of resistance including the mainstay artemisinin-based combination therapies (3), the most preferable and potent antimalarial (4). Due to these factors, the development of novel therapeutics and new target discovery is desperately needed.

In our efforts to discover new drug targets, we are considering proteases that have a role in the ubiquitin-proteasome system. Proteases have been considered promising drug targets (5) and the ubiquitin-proteasome system serves as a key pathway in protein homeostasis in eukaryotes. The 26S proteasome is a multisubunit proteolytic complex conserved in eukaryotic cells whose role is to degrade protein substrates modified by ubiquitin (Fig. 1.3) (6). Ubiquitin is a small, highly conserved protein in eukaryotes responsible for a variety of cellular functions. In the ubiquitin-proteasome pathway, ubiquitin modifies a target protein through a covalent isopeptide bond in specific lysine residues in the protein substrates. This modification is catalyzed through three successive enzymatic steps mediated by an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase. Ubiquitin itself also contains specific lysine residues used for conjugation of multiple ubiquitin monomers to generate polyubiquitin chains. Polyubiquitinated chains on a lysine-48 residue of a target protein are a common degradation signal recognized by the proteasome (7, 8) (Fig. 1.3a).

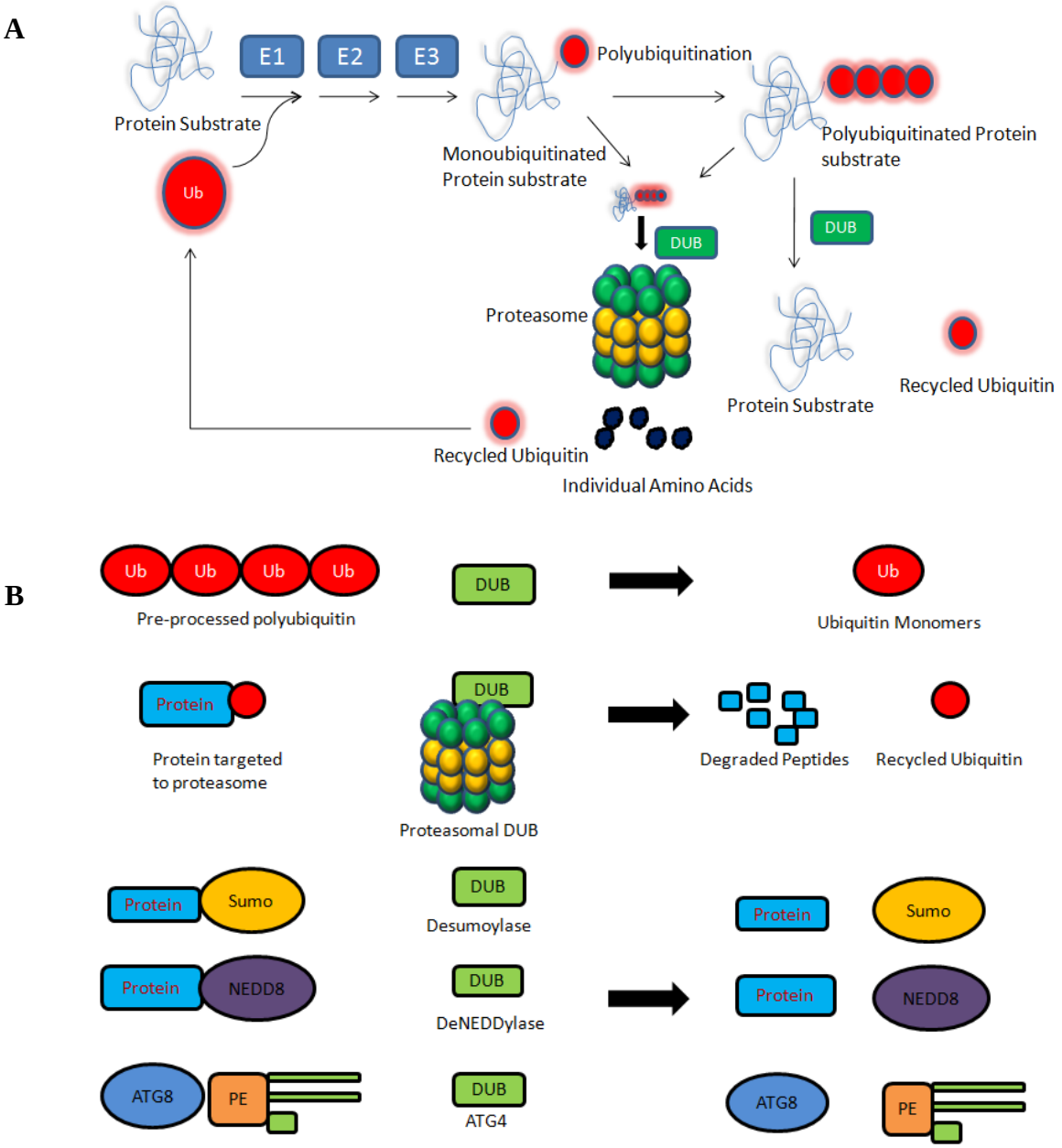

Figure 1.3 – Deubiquitinating proteases and the ubiquitin-proteasome system. (A) Covalent conjugation of ubiquitin to protein substrates is mediated by E1, E2, and E3 enzymes. Successive ubiquitination of substrates yields polyubiquitinated products. The proteasome hydrolyztes ubiquitinated substrates into individual amino acids. (B) Various functions and processes of deubiquitinating proteases and other paralogs.

Deubiquitinating proteases (DUBs) are proteases responsible for hydrolyzing the covalent protein-ubiquitin bond, reversing the actions mediated by the E1, E2, and E3 enzymes. They are thought to be involved in a variety of processes such as ubiquitin processing, ubiquitin recycling and as an antagonist to ubiquitin ligases (Fig. 1.3b) (9).

Approximately 100 putative DUBs appear to be encoded in the human genome (10), and about seventeen putative DUBs have been identified in the *P. falciparum* genome (11). Most of these PfDUBs remain uncharacterized and as yet, none have been validated as possible drug targets.

Here we use genetic approaches to manipulate and modify the *P. falciparum* genome in an effort to validate PfDUBs as drug targets by assessing their essentiality during the parasite's erythrocytic cycle. *P. falciparum* lacks RNAi capability (12); therefore the main difficulty to our goal is to specifically attenuate the protein level of each PfDUB. We have taken advantage of a recently developed gene-tagging approach which allows for tunable protein levels (13). Although such approaches are state of the art, obtaining clonal transgenic lines is difficult, requiring approximately 3 months time. In addition, we have used a similar gene-tagging strategy to purify and biochemically characterize the deubiquitinating activity for one PfDUB using an in vitro fluorogenic assay.

CHAPTER 2

PLASMDOIUM FALCIPARUM PROTEASOMAL DUBS

Ponder et al. identified seventeen putative DUBs in the *P. falciparum* genome (11). Using the identified genes, we obtained their amino acid sequences from the fully sequenced *P. falciparum* genome (14, 15) and performed a BLASTP homology search on each DUB's closest human homologs (Appendix A and C). We observed that the three proteasomal DUBs, USP14, UCH-L5, and POH1 appear to be conserved in *P. falciparum*. In mammalian systems, these DUBs associate with the 19S regulatory cap of the proteasome (Fig 2.1) to remove polyubiquitin chains from substrates prior to entering the proteasome's proteolytic site. Thus, these DUBs are likely responsible for ubiquitin removal and recycling. Due to the reported essentiality of the proteasome in *P. falciparum* (16), we are interested in the essentiality of these three associated DUBs during the parasite's erythrocytic stages.

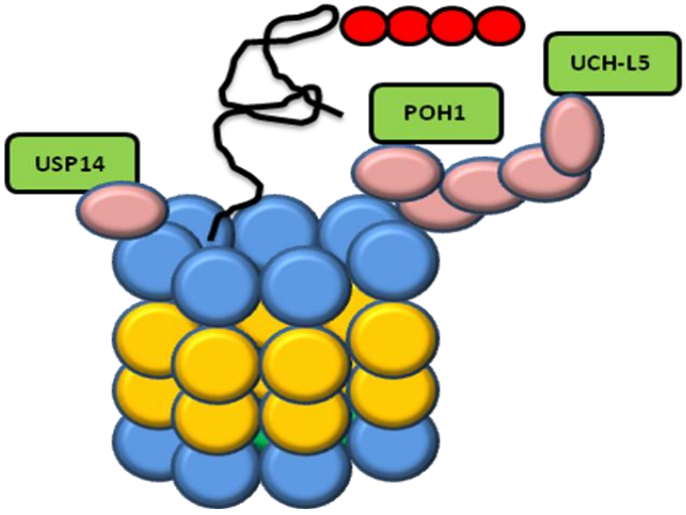

Figure 2.1 – Association of USP14, UCH-L5, and POH1 with the 19S regulatory cap (pink) of the proteasome. The alpha and beta subunits of the proteasome are labeled blue and orange respectively. Adapted from Wilkinson, et al (11).

USP14 is a cysteine protease which becomes activated upon binding to the proteasome cap (17). Deletions of USP14 in mice cause ataxia and defects associated with depletions in monomeric ubiquitin (18). Likewise, UCH-L5 (also a cysteine protease) has similar roles to USP14 in the proteasome and likely works in tandem with USP14 to trim multiple polyubiquitinated chains (19). Although UCH-L5 may have a redundant proteasomal deubiquitinating role to USP14, *P. falciparum* UCH-L5 also appears to have Nedd8 hydrolase activity, an activity not conserved in the human homolog of UCH-L5 (20). Nedd8 is a ubiquitin-like protein that post-translationally modifies the cullin family of proteins (11).

POH1 is a zinc-dependent metalloprotease which also localizes to the proteasome cap. POH1 can remove entire polyubiquitin chains from protein substrates, a function not observed for USP14 and UCH-L5 (21). POH1 has been demonstrated to be essential in yeast and humans (22) and it is thought to be a subunit of the proteasome (21).

In our genetic approach to assess the essentiality of these three putative proteasomal PfDUBs, we have used a protein attenuation system to attenuate the protein levels of each PfDUB (13). Using this system, we have generated transgenic lines of *P. falciparum* parasites in which the 3' end of the gene of interest is tagged with a gene coding for a destabilizing domain (dd). This destabilizing domain is a mutated dihydrofolate-reductase protein (DHFRdd), whose stability is dependent on the small molecule ligand trimethoprim (TMP). Removal of TMP results in misfolding and degradation of the DHFRdd (Fig. 2.2). By tagging the 3' end of the DUB gene with the coding region for DHFRdd, we are able to generate a gene which will code for a DUB-

DHFRdd fusion protein. Consequently, the abundance of a DUB-DHFRdd fusion protein can be attenuated post-translationally by removal of TMP.

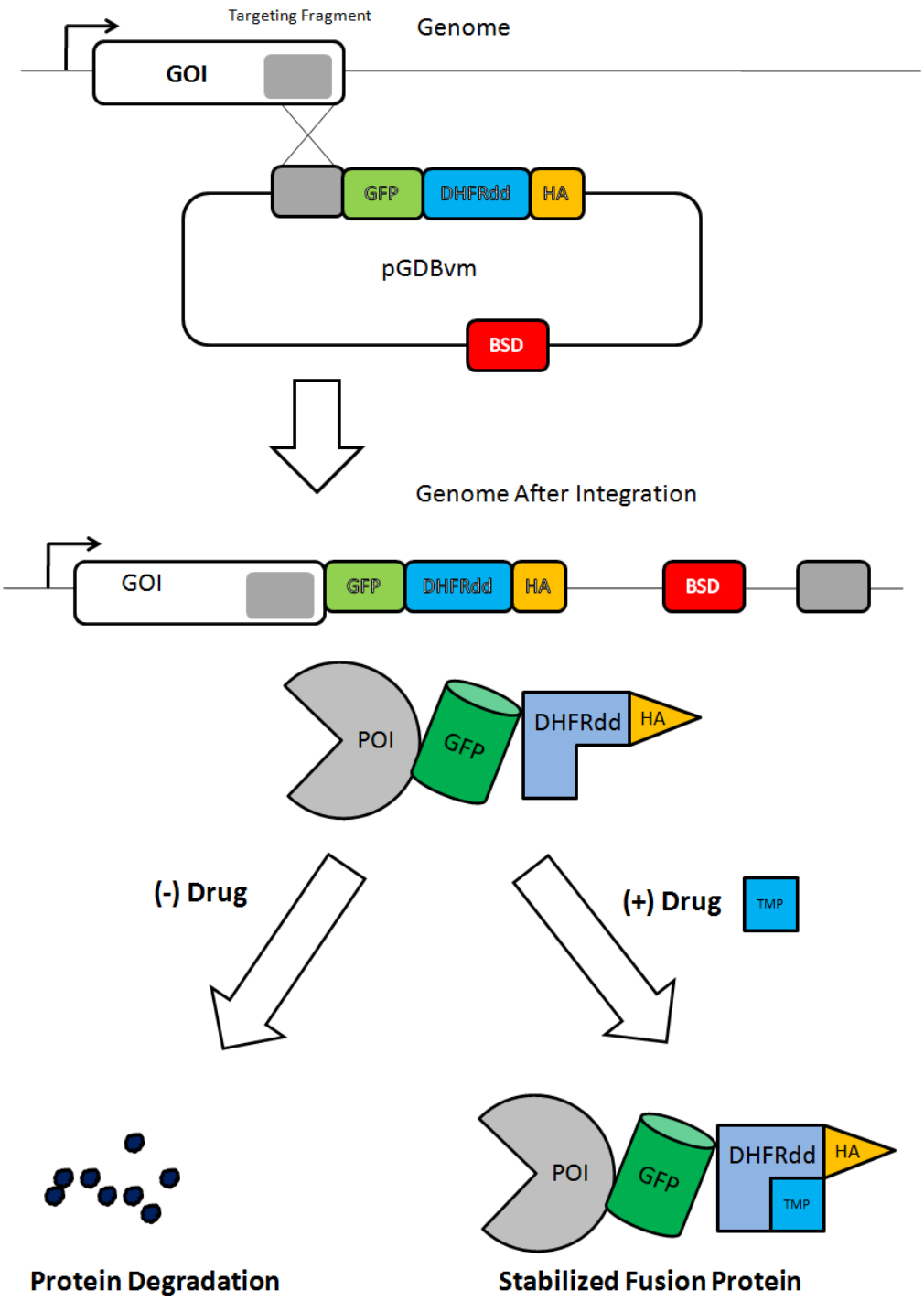

Figure 2.2 – Integration strategy of pGDBvm into the *P. falciparum* genome. The 3' end of the GOI is cloned in-frame with the GFP-DHFRdd-HA domain to generate the PfDUB-DHFRdd fusion protein whose stability is dependent on TMP.

To tag a DUB of interest with DHFRdd, we cloned a targeting fragment containing the 3' end of the gene of interest (GOI) into the pGDBvm plasmid (Muralidharan, Fig. 2.2). After successful cloning of each targeting fragment into pGDBvm, the resulting plasmids are then electroporated into *P. falciparum* parasites to undergo single crossover homologous recombination, thus tagging the native locus of the DUB (Fig. 2.2). For these experiments, all transfections were carried out in the parental line ΔPM1-3D7, a clonal, transgenic line which provides a stably integrated and expressed human DHFR gene which is resistant to TMP. We obtained clonal lines of integrant parasites using a strategy of on/off drug pressure followed by limiting dilution.

Proteasomal DUBs PfUSP14, PfUCH-L5, and PfPOH1 are expressed in P. falciparum's erythrocytic stages. Microarray data found on the PlasmoDB website suggested maximal expression for these PfDUBs during the parasite's most metabolically active trophozoite-stages (15). We therefore confirmed correct integration of our clonal lines by western blotting cell lysate of trophozoite-stage parasites. Total parasite lysate was separated by denaturing SDS-PAGE, and the fusion protein was detected using anti-HA anti-sera (Fig. 2.3 a). In addition to DHFRdd, we also successfully tagged the 3' end of PfUSP14 and PfUCH-L5 in a similar strategy with just the coding region for HA, a 1 kDa epitope tag. The observed molecular weight of each PfDUB appears to be consistent with the predicted molecular weight, suggesting these DUBs are not post-translationally modified. Additionally, primers designed to analyze integration into PfUSP14's locus confirmed integration of the plasmid by PCR, providingfurther evidence for correct integration (Fig. 2.3 b).

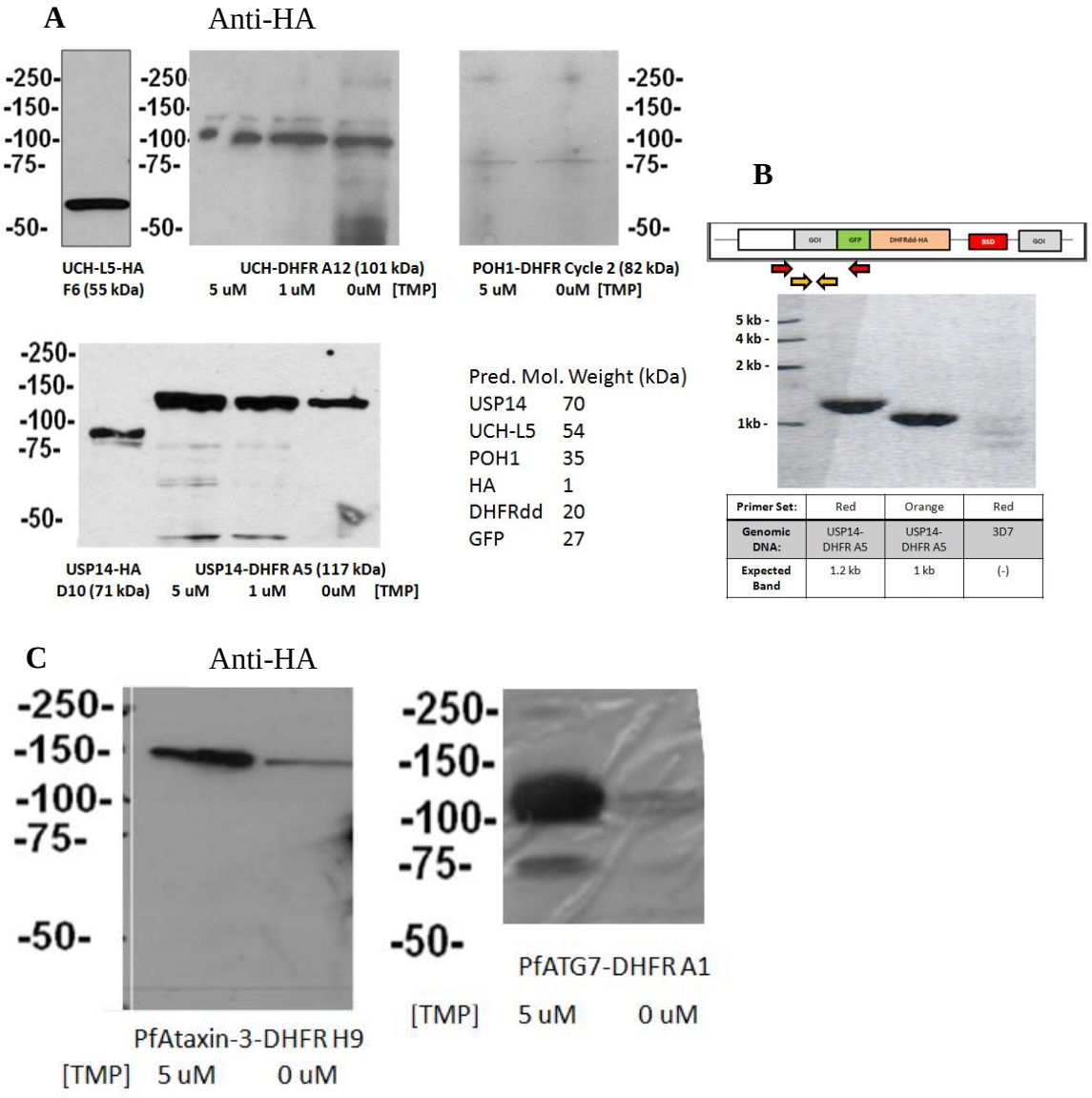

Figure 2.3 – Successful tagging of PfUSP14, PfUCH-L5, and PfPOH1 with either DHFRdd or HA. (A) Anti- HA western blot of trophozoite-stage parasites for PfUCH-L5-HA (Clone F6) and PfUSP14-HA (Clone D10) and the DHFRdd constructs: PfUCH-L5-DHFRdd Clone A12, PfUSP14-DHFRdd Clone A5, and PfPOH. Concentration of TMP is noted and reflects 48 hour treatment/removal. (B) Genotyping of PfUSP14 by PCR confirms integration into the correct locus. (C) Anti-HA western blot of trophozoite-stage parasites for PfAtaxin-3-DHFRdd (Clone H9) and PfATG7-DHFRdd (Clone A1). TMP concentration is noted and reflects 48 hour treatment/removal.

Attenuation of the Proteasomal DUB expression by removal of TMP. TMP removal should result in misfolding of the DUB-DHFRdd fusion followed by degradation by the proteasome. Attenuation of each PfDUB-DHFRdd appears to be effective after 48 hours post TMP washout, but attenuation is relatively inefficient (Fig.2.3 a). In unrelated

experiments, our lab has used this system to attenuate cellular levels of PfAtaxin-3-DHFRdd and PfATG7-DHFRdd with much greater efficiency (Fig. 2.3 c). PfAtaxin-3 and PfATG7 are independent of the ubiquitin-proteasome pathway and one possible explanation for the inefficiency in attenuating our proteasomal DUBs is that their inhibition impairs the degradation efficiency of the proteasome, which is necessary for this attenuation system. It was previously demonstrated that complete inhibition of the proteasome by lactacystin blocks replication of parasites (16). Therefore, attenuation of these PfDUBs may impair proteasome function, without the ability to inhibit it completely.

Modification of the proteasomal DUBs appears to affect parasite growth. In culturing conditions using the standard rich media RPMI 1640, we have not observed any morphological or growth phenotype of our transgenic lines. However, growth of these lines is slower than the parental ΔPM1-3D7 strain in the minimal isoleucine-only growth media (I-Media) regardless of the presence of TMP (Fig. 2.4 a-c). Although RPMI 1640 is a media that is ideal for sustained parasite growth, we used I-media to force the parasite to rely solely on its protein catabolic processes to generate amino acids (isoleucine is the only amino acid the parasite cannot obtain from catabolism of host cell hemoglobin (23)). The lack of TMP dependence is possibly due to the presence of the bulky C-terminal DHFRdd tag (~47 kDa), which might inhibit the protein's localization or enzymatic properties. Due to the inefficiency of degradation of PfUCH-L5 and PfUSP14 upon removal of TMP (Fig. 2.3a), this C-terminal tag may also disrupt normal proteasome function, which could explain the observed growth phenotype.

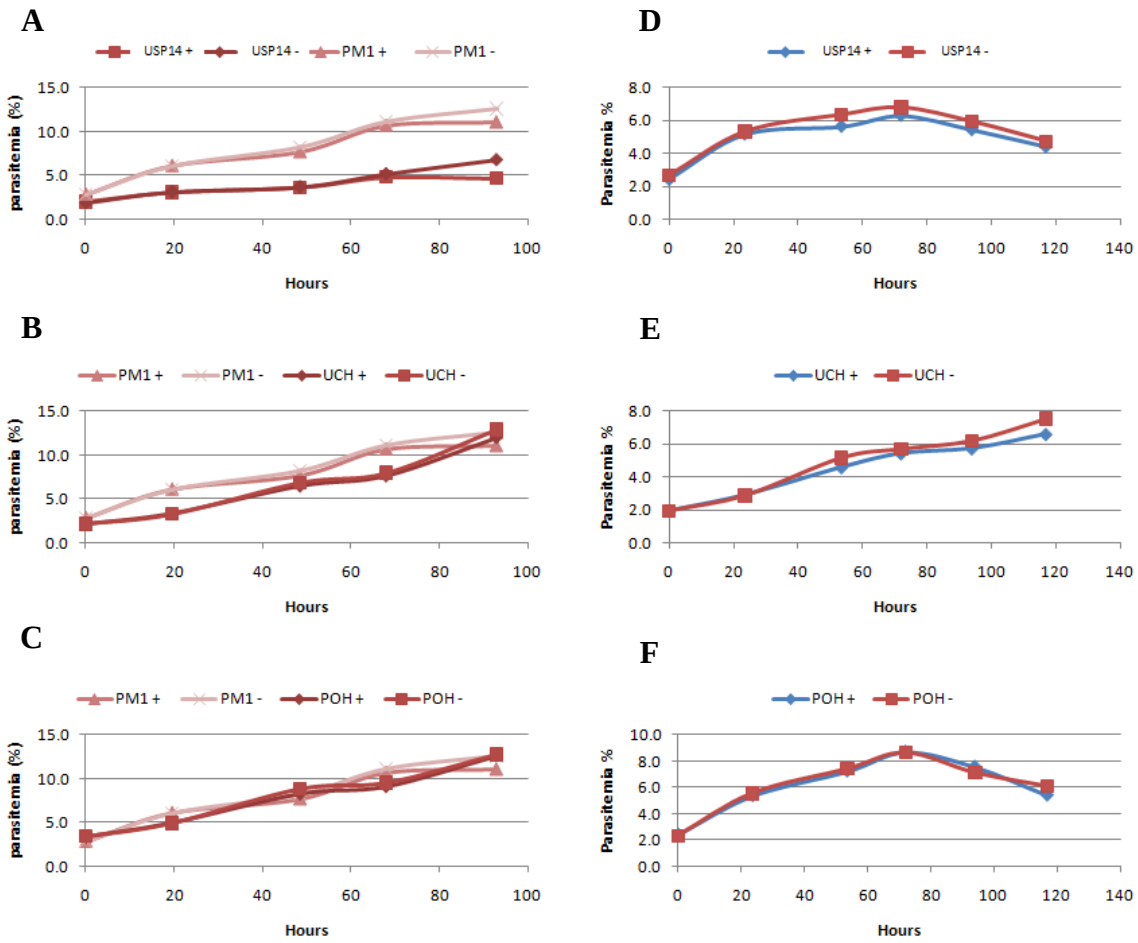

Figure 2.4 – Analysis of PfUSP14, PfUCH-L5, and PfPOH1 protein attenuation when grown in I-media. (A-C) Asynchronous cultures of PfUSP14-DHFRdd clone A5, PfUCH-L5-DHFRdd clone A12 (noted as UCH), and PfPOH1 mixed cultures were grown in the presence and absence of 0.5 µM TMP. Growth was compared with the parental ΔPM1-3D7 with similar conditions. (D-F) Analysis of synchronous PfDUB-DHFRdd cultures in the presence and absence of 0.5 µM TMP . Parasite growth was monitored by flow cytometry and cultures were grown in I-media (see Materials and Methods).

Besides a slow growth phenotype, PfUSP14-DHFRdd and PfPOH1-DHFRdd populations grown in I-media eventually die (Fig. 2.4 d and f). This suggests these DUBs to be essential under nutrient limiting conditions. In contrast, growth of PfUCH-L5 appears to stall then eventually recovers. distinguishing this PfDUB from PfUSP14 and PfPOH1, indicating it may not be essential.

It was previously observed in other systems that ubiquitin monomer depletion is deleterious for cells (24). We hypothesized that attenuation of these putative proteasomal DUBs could result in an impaired ability to recycle ubiquitin. To explore this idea, we

assessed ubiquitin monomer levels by western blotting at various concentrations of TMP (Fig. 2.5). Contrary to our hypothesis, ubiquitin monomers did not appear to be depleted in the absence of TMP in the PfUSP14-DHFRdd and PfUCH-L5-DHFRdd lines. In fact, attenuation of PfUSP14 and PfUCH-L5 levels had an opposite effect, as monomeric ubiquitin depletion appeared to increase (Fig. 2.5). Also observed was an increase in ubiquitinated proteins. This increase in ubiquitinated proteins is similar to the effects of the proteasome inhibitor MLN-273, which further suggests attenuation of PfUSP14 or PfUCH-L5 results in impaired proteasome function (25). One possible explanation for this is that the parasite can respond to reduced ubiquitin monomer pools by upregulating ubiquitin expression. A proposed experiment to determine if ubiquitin is upregulated is described in Appendix D.

The slow-growth phenotype in these experiments might be due to an indirect effect of impairment of the proteasome through a starvation related stress response. One observation that supports this idea came from our attempt to use the structural protein actin as a loading control for our attenuation experiments (Fig. 2.3 a). Surprisingly, actin levels rose as TMP concentration was reduced for both the PfUSP14-DHFRdd or PfUCH-L5-DHFRdd cell lines. An increase in ubiquitinated proteins has also been demonstrated to be a stress response (26). Therefore, it is possible the growth phenotypes observed for PfUSP14 may be due to an impaired proteasome function that causes stress. An experiment to directly assay proteasome efficiency is proposed in Appendix D.

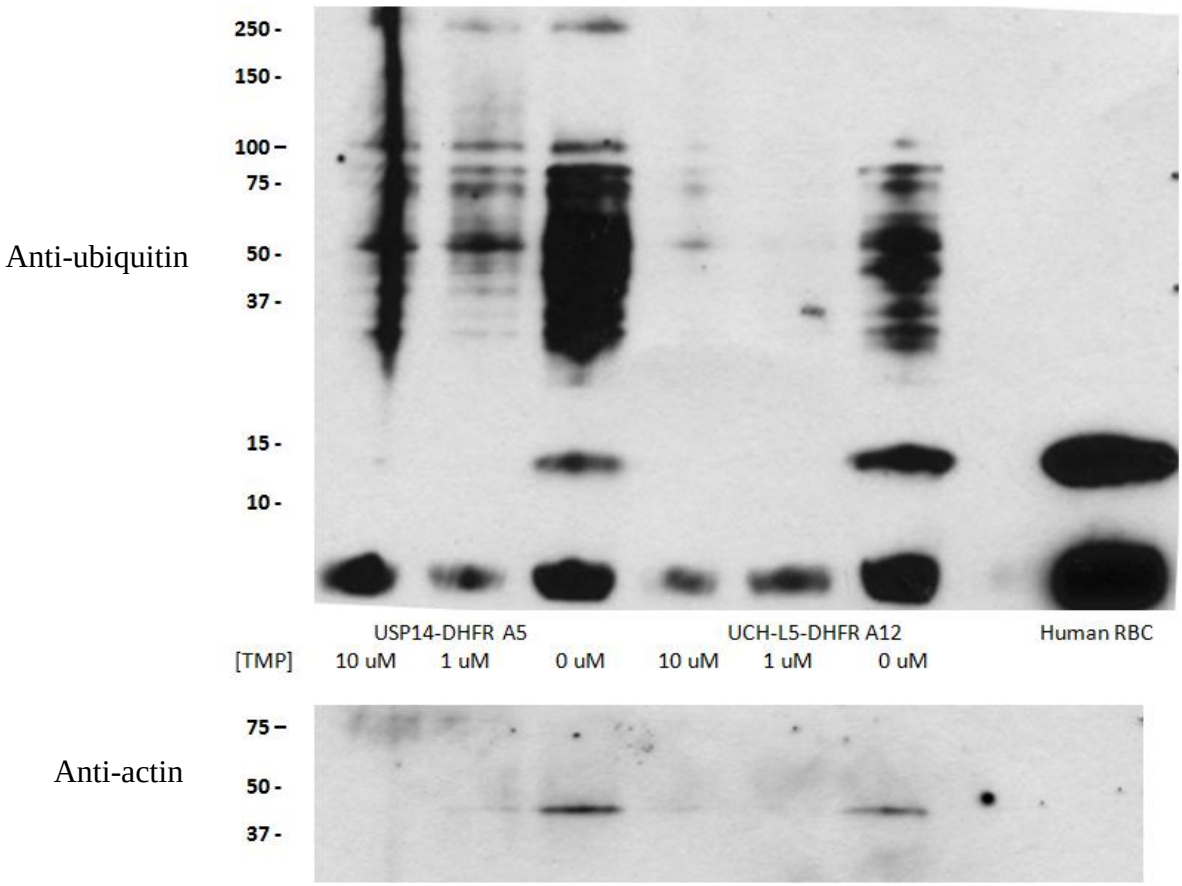

Figure 2.5 - Attenuation of proteasomal DUBs may induce stress-related response. Western blot of PfUSP14-DHFRdd clone A5 and UCH-L5-DHFR clone A12 with human ubiquitin anti-sera at varying TMP concentrations (upper panel). Membrane was stripped and was then re-probed with actin-antisera (lower panel).

A more direct possibility for the slow growth phenotype in PfUSP14-DHFRdd and PfPOH1-DHFRdd may be due to a limited supply of amino acids. Parasites in I-media are forced to rely on the protein catabolic processes of the lysosome and the proteasome. In RPMI 1640, the parasite has access to an abundant supply of exogenous amino acids from the media. If the growth phenotype is indeed attributable to amino acid starvation, could adding back cysteine, glutamine, glutamate, and methionine rescue the parasites grown in I-media? These amino acids are present minimally in hemoglobin, a major catabolic source of amino acids for the parasite (23). This experiment is currently

in progress. Additionally, phosphorylation of eIF2α, which is known to occur during amino acid starvation will be assessed by western blotting (see Appendix D) (27).

Biochemical characterization of PfUCH-L5. Using an integration strategy similar to the pGDBvm plasmid, we have tagged the 3' end of the PfUSP14 and PfUCH-L5 genes with the coding region the HA epitope to allow for purification of native protein for biochemical assays. We successfully obtained integrants in PfUSP14-HA clone A11 and PfUCH-L5 clone F6. (Fig. 2.3 a, HA tag lines). Using HA anti-sera, we immunoprecipitated each PfDUB-HA fusion protein and carried out in vitro assays for deubiquitinating activity using the fluorogenic ubiquitin substrate Ubiquitin-AMC. PfUCH-L5, like its human homolog, can hydrolyze Ub-AMC and its activity can be inhibited by the inhibitor ubiquitin-aldehyde (Fig. 2.7 a) (28). As shown, we did not observe PfUSP14 activity, which is consistent with human USP14 activity assays with Ub-AMC (29). This could suggest that Ub-AMC is not the ideal substrate for USP14 since western blotting detects presence of purified PfUSP14. We were however able to detect activity for PfUCH-L5-HA, measuring a Michaelis constant (Km) of 699.0 nM (Fig. 2.7 b). The measured Km is similar to those for PfUCH-L3 and HsUCH-L3, identified by Artavanis-Tsakonas et al of 301 nM and 162 nM respectively (30). Kcat was not calculated due to the unknown quantity of PfUCH-L5 present in the reaction.

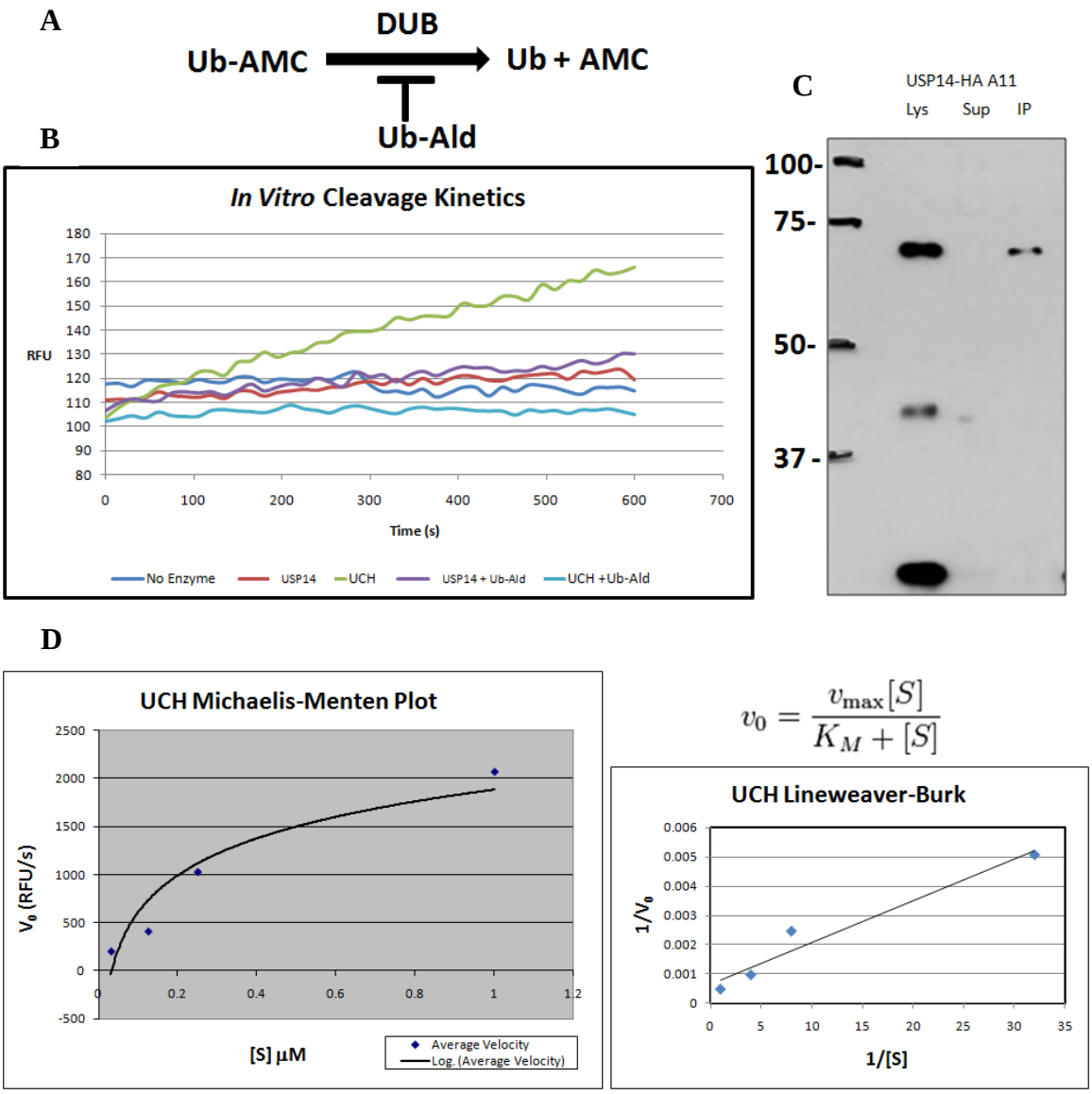

Figure 2.6 - Biochemical Characterization of PfUSP14 and PfUCH-L5. (A) Ubiquitin-AMC is a fluorogenic substrate that emits a fluorescent signal after cleavage by a deubiquitinating enzyme. Ubiquitin-aldehyde is an irreversible inhibitor of the reaction. (B) Relative florescence over time of immunoprecipitated PfUSP14-HA clone A11 and PfUCH-L5 clone F6 (noted as UCH) in the presence and absence of ubiquitin-aldehyde. (C) Anti-HA western blot of PfUSP14 lysate, supernatant, and immunoprecipitate showing presence of PfUSP14 despite no activity from (B). (D) Kinetic analysis of PfUCH-L5 determines a $K_{m.}$ of 699 nM.

CHAPTER 3

DISCUSSION

We have carried out the initial characterization of the *P. falciparum* DUBs PfUSP14, PfUCH-L5, and PfPOH1. Our studies suggest them to be associated with the proteasome, indicating their roles to be conserved in *P. falciparum* erythrocytic-stage parasites. Although the functions appear to be conserved, sequence alignments to their respective human homologs show differences in the primary amino acid sequence (Appendix C). Although these studies suggest the potential druggability of these proteases in *P. falciparum*, further modeling and structural studies are needed to address the feasibility of the design of selective inhibitors.

Due to the lack of RNAi in *P. falciparum*, our approach in evaluating the essentiality of these DUBs consisted of manipulating the parasite genome to tag the 3' end of each PfDUB gene with a sequence encoding a destabilization domain (DHFRdd). By removing the stabilizing ligand TMP, we attenuated the expression of each DUB post-translationally. Although we have evidence suggesting the essentiality of PfUSP14 and PfPOH1, a rigorous attempt to delete these genes by knockout approaches needs to be carried out. Failure to achieve a knockout in these haploid parasites would serve as the strongest evidence for essentiality.

These studies have provided us evidence of essentiality during parasite growth in minimal media. In minimal media, *P. falciparum*'s only sources of amino acids are from protein catabolic processes such as the ubiquitin-proteasome system and the lysosome. Regardless of TMP concentration, tagging of PfUSP14 and PfPOH1 with a bulky C-terminal domain may impair normal function of the PfDUB and/or the proteasome. These

transgenic parasites, when grown in I-media, die in as little as 3-4 days. This may be attributed to stress related to amino acid starvation. We will address this possibility by adding back the four additional amino acids found in limited amounts in hemoglobin, which could rescue the parasite from amino acid starvation.

Knockout of PfUSP14 and PfUCH-L5 in yeast and mice have suggested toxicity to cells resulting from ubiquitin monomer depletion. Due to their association with the proteasome, these DUBs are suggested to function in ubiquitin recycling by cleaving ubiquitin from protein substrates prior to proteasomal degradation. Thus, attenuating each PfDUB could result in a decrease of monomeric ubiquitin. However, we observed an apparent increase in monomeric ubiquitin. Additionally, there was an increase in ubiquitinated proteins in PfUSP14 and PfUCH-L5 attenuated parasites which would be consistent with impaired proteasome function. Another possibility is this increase is associated with a parasite stress response. Further studies can investigate this idea by examining if this increase in monomeric ubiquitin is due to a stress-induced upregulation of ubiquitin transcription.

Normal proteasome function may be impaired in the absence of TMP, but we have not quantitatively determined this. Western blotting of PfATG7-DHFRdd and PfAtaxin-3-DHFR dd attenuated lines (not subjects of this study) suggests degradation of these cytosolic proteins was significant after 48 hours, yet in PfUSP14 and PfUCH-L5, attenuation efficiency was less robust. This may suggest an inherent difficulty in utilizing a strategy involving proteasomal mediated degradation to examine proteins essential to proteasome function. Assays to directly measure degradation efficiency in the attenuated lines should confirm if the proteasome is truly impaired.

The growth phenotypes of PfUSP14 and PfPOH1 do, however, suggest they are essential to the parasite and that these proteases may be targets for novel antimalarial therapeutics. In our initial assays, PfUCH-L5 appears to be non-essential due to its functional redundancy to PfUSP14. This study provides, to our knowledge, the first insights into the role of the proteasomal DUBs in *P. falciparum*, a medically relevant parasitic protozoa highly reliant on protein catabolic processes. Although such pathways are conserved in human cells, these proteases have shown considerable promise as targets for anti-cancer therapeutics due to the cancerous cell's susceptibility to alterations in protein homeostasis (31). For this reason, a number of proteasomal inhibitors have been explored as anti-cancer therapeutics (bortezomib). We feel a similar situation might be true for *P. falciparum* and further studies will be carried out to assess the potential selectivity of PfDUB inhibitors possibly serving to validate these enzymes as *bona fide* drug targets for *P. falciparum*.

CHAPTER 4

MATERIALS AND METHODS

Materials

The pGDBvm plasmid was kindly obtained from Dr. Vasant Muralidharan, Washington University in St. Louis. The pM2GT-HA plasmid was obtained from Dr. Michael Klemba, Virginia Polytechnic Institute and State University.

Plasmid Construction

3D7 strain parasite genomic DNA was purified from *P. falciparum* parasites using the Blood Mini Kit (Qiagen). Primers were designed to amplify a ~1 kb fragment of the 3' end of each PfDUB and contained restriction sites for *Xho I* and *AvrII* (Appendix B). The PfDUB 3' fragment was amplified by PCR using *Taq Hi fidelity* DNA polymerase (Invitrogen) and cloned into the TOPO TA sequencing vector (Invitrogen). Each insert was confirmed by sequencing and fragments were digested with *XhoI* and *AvrII* and ligated into pM2GT-HA (Klemba) and pGDBvm (Muralidharan). Plasmids were maintained in *E. coli* DH5α (Invitrogen).

Parasite Culture and Transfection

P. falciparum (3D7 and ΔPM1-3D7 strain) parasites were cultured in human O+ erythrocytes as previously described (32) and grown at 2% hematocrit in RPMI 1640 medium enriched with gentamycin and albumax. Parasites were grown at 37°C under 5% O_2, 5% CO_2, and 90% N_2. Parasite cultures were synchronized using 5% D-sorbitol (33).

Ring stage parasites (3D7 for pM2GT-HA constructs and PM1-3D7 for pGDBvm constructs) were transfected with 100 μg of plasmid DNA using electroporation (34) and clonal integrants were obtained by cycling of drug pressure as previously described (35).

The PfPOH-DHFRdd lines used in this study were non clonal and finished 2nd cycle of drug pressure.

Parasite Purification from Human Erythrocytes

Parasites were purified from host red blood cells by incubation in a .025% saponin/phosphate-buffered saline (PBS) solution for 5 minutes followed by cold PBS washes supplemented with Complete Protease inhibitor (Roche).

Western blotting

Parasite lysate was resuspended in 1X SDS sample loading buffer, boiled for 5 min., and separated by SDS-PAGE. Loading of each lane was by equal parasite number of approximately 1x10^{7} parasites per lane. Electrophoretic transfer occurred onto a nitrocellulose membrane (GE Healthcare) 100 volts for 30 minutes. Membrane was washed and blocked using a 5% non-fat dry milk/tris-buffered saline-tween20. Rabbit anti-HA antisera (Abcam) was diluted 1:5,000 and detected by chemiluminescence using horse radish peroxidase-conjugated donkey anti-rabbit secondary (GE Healthcare) 1:10,000. Signal was visualized by exposure to X-ray film after application of an ECL kit (Supersignal West Dura, Thermo Scientific). Anti-ubiquitin western blotting used rabbit anti-human ubiquitin sera (Abcam). Anti-actin western blotting used goat anti-human actin sera (Santa Cruz) 1:2,000 and detected with a donkey anti-goat antisera (Santa Cruz) 1:5,000. Stripping of membranes was by 2% SDS-62 mM Tris (6.7pH)- 7.8uL/mL-TBS-T solution.

Parasite Growth Analysis

Parasitemia was using a high throughput sampler on a BD FacsCanto II flow cytometer. 5 μL parasite populations were resuspended in 100 μL of a 1.5 μg/mL-acridine orange-PBS solution. Parasitemias were graphed onto Microsoft Excel.

Immunoprecipitation

$8x10^8$ parasites of trophozoite-stage parasites were purified as described. Lysate was resuspended in PBS, sonicated, followed by addition of triton X-100 (1%). Anti-HA antisera and Protein G/Protein A conjugated agarose beads were added and incubated for 1 hour. Supernatant was removed and saved for western blotting. Immunoprecipitate was washed 5 times with PBS and final solution was suspended in PBS.

Ubiquitin-AMC Activity Assay and kinetic analysis

Immunoprecipitated PfDUB-HA proteins were incubated with 0.5 μM Ubiquitin-AMC (Enzo) and relative fluorescence was measured on a Spectra Max Plate Reader using excitation and emission wavelengths of 380 and 460 nM respectively (Molecular Devices). For inhibition, 0.1 μM ubiquitin-aldehyde (Enzo) was preincubated with immunoprecipitate prior to addition of substrate in the same conditions. Assay buffer consisted of 50 mM HEPES, 0.5 mM EDTA, 1 mM DTT, and 0.1 mg/mL BSA.

For kinetic analyses, PfDUB-HA was incubated at concentrations of 1 μM, 0.5 μM, 0.25 μM, 0.125 μM, and 0.0625 μM of ubiquitin-AMC. Data was imported into the PRISM software (GraphPad Software) and a K_m was calculated.

APPENDIX A

TABLE OF IDENTIFIED DUBS IN *P. FALCIPARUM*

PFALCIPARUM GENE	CLOSEST HUMAN HOMOLOG	SUB-FAMILY	DUB FUNCTION IN HUMANS	ESSENTIAL?
PF14_0576	UCH-L3	Ubiquitin C-Terminal Hydrolase	Process multimeric ubiquitin	Yes
PF11_0177	UCH-L5	Ubiquitin C-Terminal Hydrolase	Deubiquitinating and deneddylating activity; associates with proteasome	Unknown
MAL7P1.147	USP7/HAUSP	Ubiquitin Specific Protease	P53 and Mdm2 deubiquitination.	Unknown
PFA0220w	USP36	Ubiquitin Specific Protease	Unknown	Unknown
PFD0680c	USP5/Isopeptidase T	Ubiquitin Specific Protease	Process ubiquitin chains to recycle ubiquitin	Unknown
PFE1355c	USP14	Ubiquitin Specific Protease	Disassembles polyubiquitin chains at proteasome; ubiquitin recycling	Unknown
PFE0835w	USP4	Ubiquitin Specific Protease	Rho52 deubiquitination.	Unknown
PFI0225w	USP42	Ubiquitin Specific Protease	Unknown	Unknown
PF13_0096	USP39	Ubiquitin Specific Protease	Unknown	Unknown
PF14_0145	UCH36	Ubiquitin Specific Protease	Unknown	Unknown
PFL1295w	Ataxin-3	Josephin Domain	Deubiquitinates K63-ubiquitinated proteins	Unknown
MAL13P1.343	POH1/Proteasome Subunit 14	Jab1/MPN/Mov34 Metalloenzyme	Catalyzes release of polyubiquitin chains at proteasome; subunit of proteasome	Unknown
PFL0865w	PPPDE		Unknown	Unknown
PFL1635w	Sumo/sentrin Specific Peptidase 1	Desumoylase	Desumoylating activity	Unknown
MAL8P1.157	Ulp2 (S. pombe)	Desumoylase	Desumoylating activity	Unknown
PF10_0092	Wss1p (S. cerevisiae)	Metalloprotease	Unknown metalloprotease	Unknown
PF14_0171	ATG4	ATG8 C-Terminal Hydrolase	Autophagy pathway	Unknown

APPENDIX B

PRIMERS USED TO CLONE EACH PFDUB GENE

PfUSP14	For : CTCGAGGGAAACTGTTGAACAACATG Rev: CCTAGGTTCCTTAATATCCATATTCAT
PfUCH-L5	For: CTCGAGTGCATGTGCTACACAAGCTA Rev: CCTAGGCGAAATCATAAATGTTTCCT
PfPOH1	For: CCTTCTCGAGAGTACCTATGGAAGTT Rev: CCTCCCTAGGAAAGAACAGAGTATTTG

APPENDIX C

PFDUB SEQUENCE ALIGNMENTS WITH HUMAN HOMOLOGS

USP14

```
PfUSP14         MTLVNITVKWKNQVFNNIELDVSEPLILLKTQLWQLTNVPPEKQKLMYKGLLKDDVDLSL 60
HsUSP14         MPLYSVTVKWGKEKFEGVELNTDEPPMVFKAQLFALTGVQPARQKVMVKGGTLKDDDWGN 60
                *.* .:**** :: *:.:**:..** :::*:**: **.* * :**:* **   .* * .

PfUSP14         LNIKENDKIMLVGSAESLVEKP-KDIIFEEDLTNEEKQKIHTKENIIFEEQGIVNLGNTC 119
HsUSP14         IKIKNGMTLLMMGSADALPEEPSAKTVFVEDMTEEQLASAMELP------CGLTNLGNTC 114
                ::**:. .::::***::* *:*  . :* **:*:*:  .           *:.******

PfUSP14         YFNAVLQFLTSFDDLGNFLRSYK-SKESKLIKTNKDILFDSFIEFAHSFEKSSEPYVPVT 178
HsUSP14         YMNATVQCIRSVPELKDALKRYAGALRASGEMASAQYITAALRDLFDSMDKTSSSIPPII 174
                *:**.:* : *. :* : *: *  : .:.   :. : :  :: :: .*::*:*..  *:

PfUSP14         LLKSFRDVYPKFKSVNLRTKQYAQQDAEECMNAILTCLNEQTDNKIIDKLFSFQIISNMK 238
HsUSP14         LLQFLHMAFPQFAEKGE-QGQYLQQDANECW--------------------------IQ 206
                **: :: .:*:* . .    ** ****:**                           ::

PfUSP14         FVETVEQHEQKEKKDEKKDEKKDEKKDEKKEEKKEEIKNINITSNNNNIDNNNDNNDNNN 298
HsUSP14         MMRVLQQKLEAIEDDSVKETDSSSASAATPSKKKSLIDQFFGVEFETTMKCTESEEEEVT 266
                ::..::*: :  :.*. *: .... .  . .:**. *.::  .. :..:. .:.:::: .

PfUSP14         NNNNNSVQQNDHNNKDISHNNIFETTQEFNNKLICYMGTPNTPVNHLHEGIRLSLHEKIR 358
HsUSP14         KGKENQLQLSCFIN--------------------------QEVKYLFTGLKLRLQEEIT 299
                :.::*.:* . . *                           *::*. *::* *:*:*

PfUSP14         KNRNEDNKECIYEKKSEINSLPPYLIVHFLRFESKKIVESNNSGVSVVTAKICRKVSFPD 418
HsUSP14         KQSPTLQRNALYIKSSKISRLPAYLTIQMVRFFYKEKESVN--------AKVLKDVKFPL 351
                *:    :::.:* *.*:*. **.** ::::**  *:  . *        **: :.*.**

PfUSP14         TFDMYDFCSEKIKEELKIARDIIMKRKDKETSLSPQKENIQNIEQNNINNQYNQNNSNDN 478
HsUSP14         MLDMYELCTPELQEKMVSFR-----------------SKFKDLEDKKVNQQPNTSDKKSS 394
                 :***::*: :::*::   *                 .::::::::::*:* * .:.:..

PfUSP14         PKHVQEHTNQINKEELIELPTGEYELISVITHKGRNEESGHYIAWKKMKKFFSSNSNIDQ 538
HsUSP14         PQKEVKYEPFSFADDIGSNNCGYYDLQAVLTHQGRSSSSGHYVSWVKRK----------- 443


PfUSP14         NESSNKKTKNANDSLWLKMDDDKVSTHKFSSIDFYGGCSDYNIAVLLLYKRKNISCTPDE 598
HsUSP14         ------------QDEWIKFDDDKVSIVTPEDILRLSGGGDWHIAYVLLYGPRRVEIMEEE 491
                           :. *:*:******  . ..*   .* .*::** :***  :.:.   :*

PfUSP14         MNMDIKE 605
HsUSP14         SEQ---- 494
                 :
```

UCH-L5

```
PfUCH-L5        MARDNENILEEWCLIESNPCIFYDMLKR-----MGATEISVEDVYSLSYFDDYINNKEII 55
HsUCH-L5        MSLGWLERPPALSRAAGDGARRLSGSRRGDVWLTSSAAGLLRSVAGGSWCGGQLRARGGS 60
                *: .  :     .   .: .   .  :*      .::   :..* . *: .. :. :

PfUCH-L5        NMNHILGVDTYLGENNKTLDKENNVVDVIELYKNNICMEDKYN----KLLKHHSYIYGII 111
HsUCH-L5        GRCVARAMTGNAGEWCLMESDPGVFTELIKGFGCRGAQVEEIWSLEPENFEKLKPVHGLI 120
                .     .:    **     .. . ..::*: :  . .  ::      : ::: . ::*:*

PfUCH-L5        FLFNIG-KHYKNNKYIEHNVPDNLFFAKQVIPNACATQAILSIVLN---KDIELNDEIKN 167
HsUCH-L5        FLFKWQPGEEPAGSVVQDSRLDTIFFAKQVINNACATQAIVSVLLNCTHQDVHLGETLSE 180
                ***:    .   .. ::..  *.:******* ********:*::**   :*:.*.: :.:

PfUCH-L5        IKTFSLNFDSSMKGLTLSNCTFLRNIHNSYKPPIYLDKEDVHHDKKKSEDSFHFVSYISF 227
HsUCH-L5        FKEFSQSFDAAMKGLALSNSDVIRQVHNSFARQQMFEFDTKTSAKE--EDAFHFVSYVPV 238
                :* ** .**::****:***. .:*::***:     :: :     *:  **:******:..

PfUCH-L5        QDKVYLLDGLQSGPVLINADEQNKPNPNNNNNNKDNDNDNNNNNNNNNNNNNNNNNNNN 287
HsUCH-L5        NGRLYELDGLREGPIDLGACNQD------------------------------------ 261
                :.::* ****:.**: :.* :*:

PfUCH-L5        NNNNNIGMNGKDWIEISREHIKKEIDEICNSQTNNDVRFNIIAVMKDKEYIIQEYINIHR 347
HsUCH-L5        -----------DWISAVRPVIEKRIQKYS----EGEIRFNLMAIVSDR------------ 294
                           ***.  *  *:*.*:: .    :.::***::*::.*:

PfUCH-L5        IVKQRVNIKLINLGENIELSDEINEDEFPLLNDIPSIENLPNNVDTLYNIVNKSTLEINY 407
HsUCH-L5        --------KMIYEQKIAELQRQLAEEE-------------PMDTDQGNSMLSAIQSEVAK 333
                        *:*   :  **. :: *:*             * :.*   .::.    *:

PfUCH-L5        LQSLLHEQKEIKKLWNKELTFKFFNFYPFIMSSLNLMAKHKLLKDAYQKEKLKNATKS-- 465
HsUCH-L5        NQMLIEEEVQKLKRYKIENIRRKHNYLPFIMELLKTLAEHQQLIPLVEKAKEKQNAKKAQ 393
                 * *:.*: :  * :: *   : .*: ****. *: :*:*: *    :* * *: :*.

PfUCH-L5        ---
HsUCH-L5        ETK 396
```

POH1

```
PfPOH1          MAGIPSSLRELFYSFSDGNGMNNETLADTSEQVYISPLALLKILKHGRAGVPMEVMGLML 60
HsPOH1          MDRLLR-LGGGMPGLGQGP-PTDAPAVDTAEQVYISSLALLKMLKHGRAGVPMEVMGLML 58
                *  :   *   : .:.:*   .: . .**:******.*****:*****************

PfPOH1          GEIVDEYTIRIVDVFAMPQSGNSVSVEAVDPVYQTNMLEELKKTGRHEMVVGWYHSHPGF 120
HsPOH1          GEFVDDYTVRVIDVFAMPQSGTGVSVEAVDPVFQAKMLDMLKQTGRPEMVVGWYHSHPGF 118
                **:**:**:*::*********..*********:*::**: **:*** ************

PfPOH1          GCWLSGTDVNTQKSFEQLNPRTIGVVVDPIQSVKGKVVIDCFRLINPHILMLGQEPRQTT 180
HsPOH1          GCWLSGVDINTQQSFEALSERAVAVVVDPIQSVKGKVVIDAFRLINANMMVLGHEPRQTT 178
                ******.*:***:*** *. *::.***************.*****.::::**:******

PfPOH1          SNIGYLTKPTLTALVHGLNRNYYSIVINYRKNELEKNMLLNLHKDMWTNPLKLNDFHEQK 240
HsPOH1          SNLGHLNKPSIQALIHGLNRHYYSITINYRKNELEQKMLLNLHKKSWMEGLTLQDYSEHC 238
                **:*:*.**:: **:*****:****.*********::*******. * : *.*:*: *:

PfPOH1          KSSDETLEDIKKLTTLYNKNLRNEMKKTSEEILLENIGKIDAKKRIQNSVETLLNESILT 300
HsPOH1          KHNESVVKEMLELAKNYNKAVEEEDKMTPEQLAIKNVGKQDPKRHLEEHVDVLMTSNIVQ 298
                * .:..:::: :*:. *** :.:* * *.*:: ::*:** *.*::::: *:.*:...*:

PfPOH1          CIGTMANTLFF- 311
HsPOH1          CLAAMLDTVVFK 310
                *:.:* :*:.*
```

APPENDIX D

UNANSWERED QUESTIONS AND PROPOSED EXPERIMENTS

Is ubiquitin upregulated in response to disruption of ubiquitin recycling?

Our results from the anti-ubiquitin western blot (Fig. 2.5) suggest that there was not a depletion of ubiquitin monomers in our PfUSP14-DHFRdd and PfUCH-L5-DHFRdd attenuated lines, contrary to our hypothesis. However, this does not reject our hypothesis, as ubiquitin could be upregulated in response to stress. In order to determine if ubiquitin is upregulated transcriptionally, quantitative PCR or northern blotting can confirm if the RNA levels of ubiquitin is increased in the stressed PfDUB attenuated lines.

Are the growth phenotypes observed due to impaired proteasome function?

We have some evidence of impaired proteasome function due to the incomplete degradation of PfUSP14 and PfUCH-L5 and an accumulation of ubiquitinated-proteins. However, we do not have quantitative or kinetic data to confirm impaired function. One approach includes purification of *P. falciparum* proteasomes by immunoprecipitation and comparing activity in the PfDUB attenuated lines and wild type lines. This assay is dependent on the identification of a substrate whose degradation can be monitored by fluorescence for example.

Alternatively, we can also assay if sensitivity to known proteasome inhibitors is affected. Using a known inhibitor such as epoxomicin or bortezomib, we can determine if the IC_{50} values of these inhibitors are decreased from wild type strains, suggesting an increase in proteasome sensitivity.

Is the phenotype with PfUSP14 associated with a bulky C-terminal adduct?

PfUSP14-DHFRdd lines stalled and eventually died in I-media regardless of the presence of TMP. To assess if PfUSP14's normal function is inhibited by the C-terminal adduct, an activity assay will be performed. However, this assay depends on the identification of a substrate for PfUSP14 since we have determined that it cannot cleave Ub-AMC.

Likewise, the bulky C-terminal adduct may be inhibiting localization into the proteasome, which could answer why PfUSP14-DHFRdd lines grew slower regardless of the TMP concentration. To test this, we can use fluorescence microscopy to determine if they co-localize. A non-essential proteasome subunit can be tagged with a different fluorescent reporter such as RFP and expressed episomally. We can now determine co-localization since there is a GFP domain in the pGDBvm plasmid.

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